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# Angiotensin II-dependent persistent podocyte loss from destabilized glomeruli causes progression of end stage kidney disease

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Podocyte depletion is a major mechanism driving glomerulosclerosis. Progression is the process by which progressive glomerulosclerosis leads to end stage kidney disease (ESKD). In order to determine mechanisms contributing to persistent podocyte loss, we used a human diphtheria toxin transgenic rat model. After initial diphtheria toxin-induced podocyte injury (over 30% loss in 4 weeks), glomeruli became destabilized, resulting in continued autonomous podocyte loss causing global podocyte depletion (ESKD) by 13 weeks. This was monitored by urine mRNA analysis and by quantitating podocytes in glomeruli. Similar patterns of podocyte depletion were found in the puromycin aminonucleoside and 5/6 nephrectomy rat models of progressive end-stage disease. Angiotensin II blockade (combined enalapril and losartan) restabilized the glomeruli, and prevented continuous podocyte loss and progression to ESKD. Discontinuing angiotensin II blockade resulted in recurrent glomerular destabilization, podocyte loss, and progression to ESKD. Reduction in blood pressure alone did not reduce proteinuria or prevent podocyte loss from destabilized glomeruli. The protective effect of angiotensin II blockade was entirely accounted for by reduced podocyte loss. Thus, an initiating event resulting in a critical degree of podocyte depletion can destabilize glomeruli and initiate a superimposed angiotensin II-dependent podocyte loss process that accelerates progression resulting in eventual global podocyte depletion and ESKD. These events can be monitored noninvasively in real-time through urine mRNA assays.

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Compelling evidence now supports the concept that a major cause of glomerulosclerosis is podocyte injury and depletion from glomeruli.<sup>1–33</sup> However, the mechanism of progression to end-stage kidney disease (ESKD) is poorly understood. Progression of glomerular diseases is the mechanism by which cumulative glomerular injury results in progressive loss of kidney function eventually resulting in ESKD. It is manifest by an increasing proportion of glomeruli becoming sclerotic over time and with downstream-associated loss of renal tubules and their replacement by interstitial fibrosis. As the major cause of glomerulosclerosis is podocyte injury and depletion, and progression is due to progressive glomerulosclerosis, we hypothesized that progression may be caused by continual podocyte loss over time, which in turn results in progressively more glomerulosclerosis, leading eventually to global podocyte depletion and ESKD. We previously reported data to support this general concept through urine messenger RNA (mRNA) assays developed to monitor podocyte loss.<sup>33</sup> However, if the continuous podocyte depletion hypothesis for progression is correct, then it must also explain how angiotensin II blockade ameliorates progression. Rigorous testing of this hypothesis is central to understanding the progression process and developing improved strategies for monitoring and preventing progression in the clinic.

## RESULTS

### Persistent podocyte depletion following initiation of injury in the hDTR Fischer 344 rat model

Initiation of glomerular injury was induced by intravenous injection of diphtheria toxin (DT, 25 ng/kg) into human diphtheria toxin receptor (hDTR) transgenic rats to deplete 30–40% of podocytes by 4 weeks (Figure 1a–c). The glomerular podocyte complement was measured by estimating WT1-positive nuclei per glomerulus (Figure 1b), and by measuring podocyte area as a percentage of the glomerular tuft area using the podocyte-specific marker GLEPP1 (glomerular epithelial protein 1) using immunoperoxidase staining (Figure 1c). *A priori* one would expect that following an episode of podocyte depletion there would be

time-limited development of proteinuria and podocyte loss, followed by a healing phase associated with glomerulosclerosis proportional to the amount of initial podocyte depletion. However, this is not what we observed. Sequential kidney biopsies at 4, 8, and 13 weeks revealed that the glomerular podocyte complement continued to autonomously decrease long after the initial insult was over, so that progressively fewer podocytes remained at successive time points as assessed by the two independent quantitative methods (Figure 1b and c). Thus, an initial podocyte depletion event resulted in apparent glomerular destabilization such that glomeruli continued to lose podocytes over time in spite of no further initiating injury. By 13 weeks, glomeruli had become globally depleted of podocytes in association with marked glomerulosclerosis, interstitial fibrosis, and ectatic tubules typical of the end-stage kidney.

Measurement of specific podocyte markers (nephlin and podocin) in urine allowed daily noninvasive assessment of podocyte loss in the model (Figure 1d). Initial acute podocyte injury was accompanied by increased nephlin, as well as podocin mRNAs in urine. Persistent podocyte loss during the chronic progression phase from 4–13 weeks was associated with high levels of podocin mRNA, but not nephlin mRNA, in urine as previously reported.<sup>33</sup> This difference in expression of two podocyte-specific markers is conveniently captured as the podocin/nephlin mRNA ratio. The continuously elevated urine podocin and podocin/nephlin mRNA ratios in the chronic phase of progression in this model therefore reflect continuous podocyte loss from the destabilized glomerulus. Thus, we can state from this experiment that an initial episode of podocyte injury causing 30–40% podocyte depletion over the acute injury period (up to 4 weeks) resulted in further progressive chronic podocyte loss over time as measured by three different methods (podocyte tuft number, podocyte area in the tuft, and urine podocyte mRNA measurements).

To understand the level of podocyte injury required to trigger persistent podocyte loss, a second experiment was conducted using variable amounts of initial podocyte depletion. When the initial podocyte depletion by 4 weeks at biopsy was <20%, then progressive loss of podocytes did not occur. In contrast, when initial podocyte depletion was high (>60%), rats reached ESKD before 8 weeks (Figure 2).

Figure 3a shows the time course of additional biomarkers including aquaporin 2 mRNA as a control tubular biomarker, which did not change throughout the time course, except to decrease toward ESKD in association with reduced residual nephron mass. Urine transforming growth factor (TGF) $\beta$ 1 mRNA was used as a biomarker for the profibrotic milieu associated with progression and increased as expected. Control values for each parameter measured over the time course of the progression process are also provided in Figure 3a.

In control studies (Supplementary Figure S1 online), we evaluated the effect of proteinuria *per se* on the urine mRNA biomarkers in the short term using the bovine serum

albumin protein overload rat model. Increasing the urine protein/creatinine ratio to >80 caused by bovine serum albumin injection into the peritoneal cavity of rats did not significantly change urine podocin, podocin/nephlin ratio, or TGF $\beta$ 1 mRNA urine biomarkers. In contrast, a proximal tubular cationic amino acid transporter (Slc7a13) mRNA increased >100-fold in association with proteinuria. These data show that the changes in urine mRNA biomarkers observed were not a nonspecific consequence of proteinuria *per se*.

We conclude that in the hDTR rat model an initiating episode of podocyte depletion above a threshold level (~30%) can promote autonomous long-term podocyte loss from glomeruli, resulting eventually in global podocyte depletion and ESKD, and that this process can be monitored noninvasively through urine podocyte mRNAs.

#### **Persistent podocyte loss and progression in the puromycin aminonucleoside model**

To confirm that the conclusions drawn in the hDTR transgenic Fischer 344 rat were reproducible in another model system, we induced progression in Sprague–Dawley (SD) rats using puromycin aminonucleoside (PAN). Results are shown in Figure 3b and Supplementary Figure S2 online. Progression to ESKD was associated with persistent podocin mRNA loss in the urine. High levels of nephlin were only present in the acute phase after PAN injection (as previously found for the hDTR rat model). The urine podocin/nephlin ratio remained elevated throughout the progression time course. By the time rats reached ESKD, glomeruli were globally depleted of podocytes as previously observed for the hDTR model. Similar changes in urine podocyte and TGF $\beta$ 1 mRNAs were also present as previously seen with the hDTR model system. Urine AQP2 was increased in the acute phase (after PAN injection), which could reflect a direct toxic effect of PAN on tubules themselves (Figure 3b). In the progression phase, similar patterns of urine mRNAs were present in the PAN model in the SD rats as had been documented with the hDTR model in the Fischer 344 rats.

#### **Persistent podocyte loss and progression in the 5/6 nephrectomy model**

The hDTR and PAN models are both initiated by direct podocyte injury. Progression in the 5/6 nephrectomy model is initiated by removal of 5/6ths of kidney mass, thereby resulting in remaining kidney hypertrophy and progression to ESKD over time. To determine whether podocyte depletion occurs in this model, we measured the same parameters as those used in the two previous models. As shown in Figure 3c and Supplementary Figure S3 online, the same stigmata of progression were seen in the 5/6 nephrectomy model as in the hDTR and PAN models. Progression was associated with persistent loss of podocytes from glomeruli as measured by an elevated level of urine podocin and podocin/nephlin mRNA ratios and persistently high urine TGF $\beta$ 1 mRNA levels, proteinuria, development of

marked hypertension, and a histological appearance of glomerular enlargement, patchy loss of podocytes from glomeruli, focal segmental glomerulosclerosis, and interstitial scarring on Masson's trichrome staining.

Data from three models in two rat strains are compatible with the conclusion that progression is driven by persistent podocyte loss over time, resulting in progressive glomerulosclerosis and secondary interstitial fibrosis.

### Prevention of autonomous podocyte loss by angiotensin II blockade

To test the hypothesis that angiotensin II blockade might mitigate autonomous podocyte loss observed in the hDTR model, we used a combination of an angiotensin converting enzyme inhibitor (enalapril at 10 mg/kg/day) and an angiotensin II receptor blocker (losartan at 50 mg/kg/day) delivered in drinking water. Drug treatment was not started until 5 days after intravenous DT injection in order to ensure that DT access to podocytes would not be affected by drug effects on glomerular filtration rate. Figure 4 shows that the drug combination reduced proteinuria starting 5 days after initiation of drug treatment, reflecting hemodynamic effects of the drug on glomerular filtration of proteins. Blood pressure was also reduced in a similar time frame. Thus, there was a robust impact of drug treatment as assessed by physiological parameters. However, it was not until after day 21 (16 days after the start of drug treatment) that there was a measurable impact of drug on reducing urine podocyte mRNA levels for both nephrin and podocin relative to non-drug-treated rats. This time lag suggests that time-dependent biological processes other than solely hemodynamic factors may be responsible. After day 21, angiotensin II blockade had a marked and persistent effect on reducing the urine podocyte biomarker levels including the urine podocin/nephrin mRNA ratio through 9 weeks after initiation of injury.

Figure 4a shows representative histological analysis of drug-treated and non-drug-treated kidneys. The upper panels demonstrate that the non-drug-treated rats had few podocytes remaining in glomeruli by 9 weeks, whereas drug-treated rat glomeruli had only minor focal and segmental patchy loss of podocytes from glomerular tufts detected by GLEPP1 peroxidase staining. Masson's trichrome-stained

sections demonstrated typical features of the end-stage kidney with glomerular sclerosis and interstitial fibrosis in non-drug-treated rats. In contrast, drug-treated rats had only small areas of focal and segmental glomerulosclerosis with glomeruli remaining largely intact and the interstitial compartment being essentially normal.

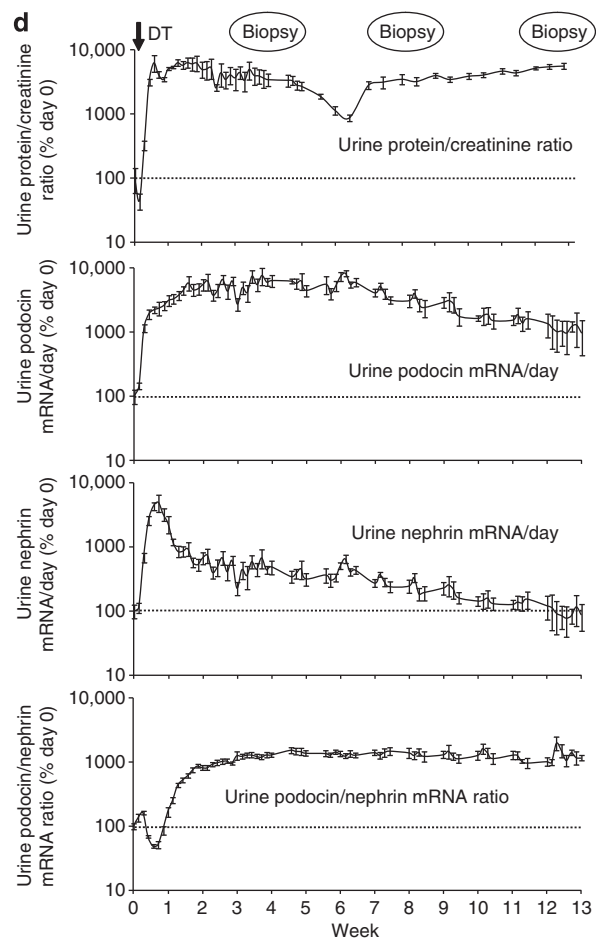
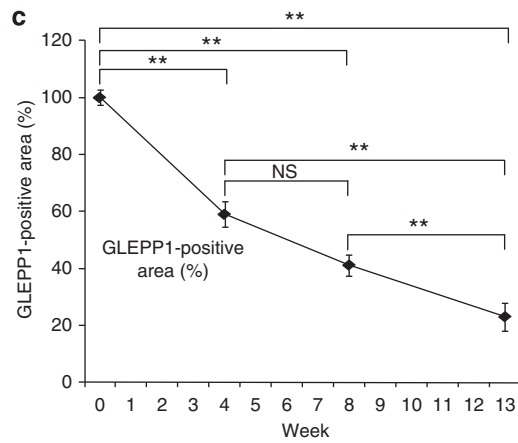
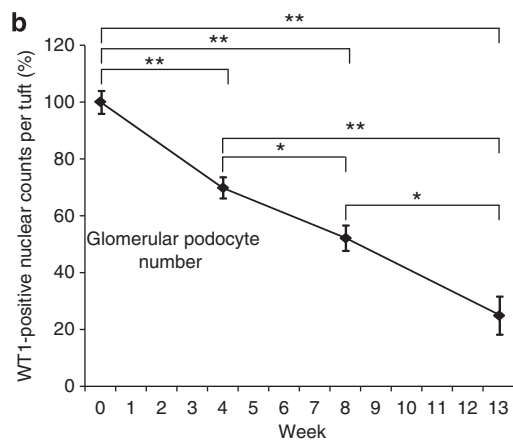
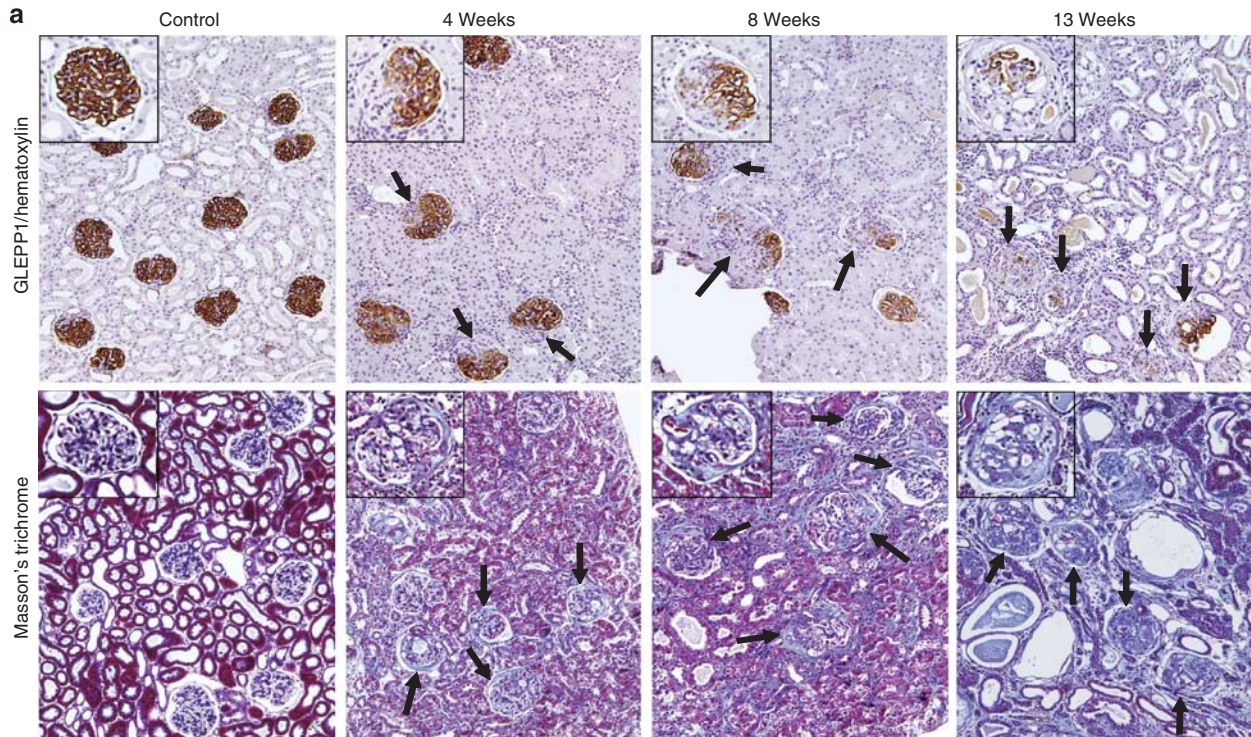
Figure 4b and c documenting quantification of the podocyte complement by two methods showed that drug-treated rats had only lost about 30% of their podocytes following DT injection, as compared with loss of 70–80% in non-drug-treated rats (Figure 4b and c). Quantification of the urine podocin mRNA loss showed a similar reduction in podocyte loss in the drug-treated rats as was seen by histological methods, thereby confirming that the three methods of monitoring podocyte loss were congruent.

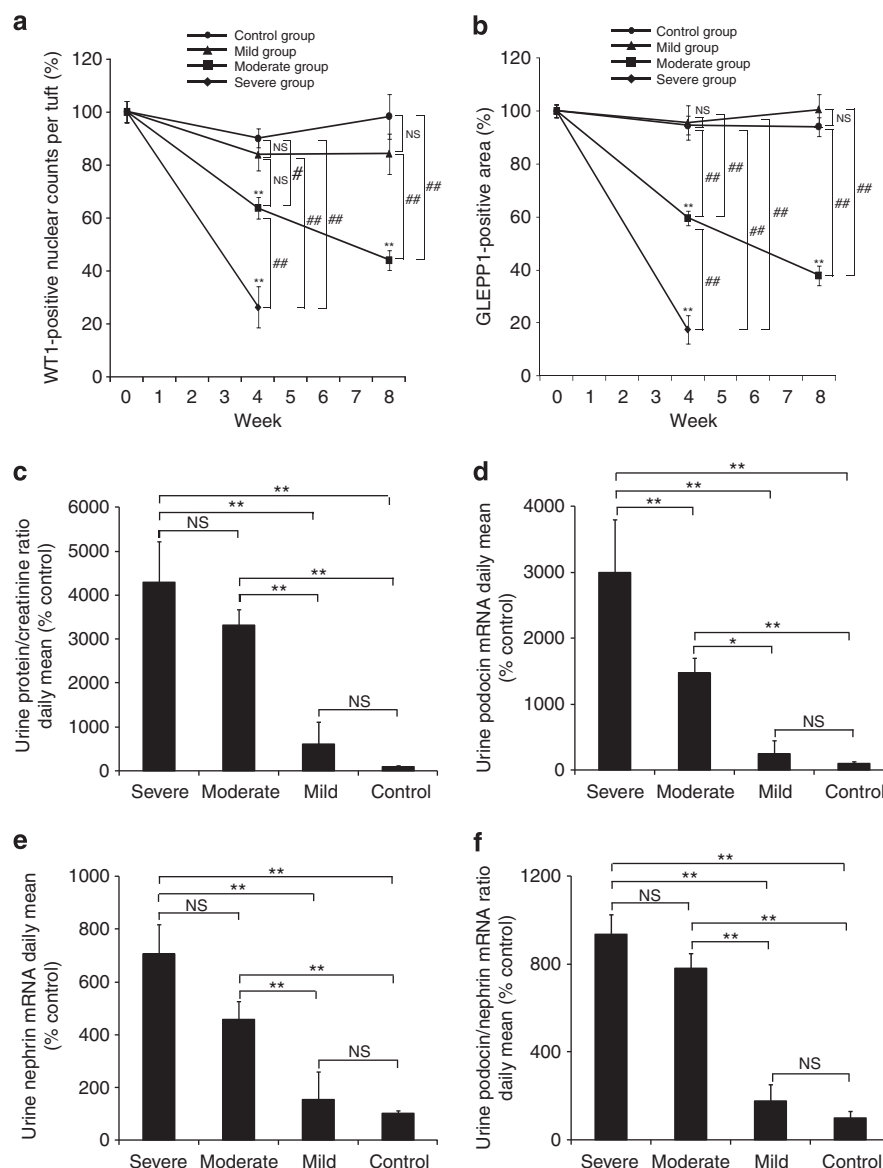
From this result, we concluded that glomeruli that were destabilized by a critical degree of podocyte depletion, so they would continue to lose podocytes over time until ESKD (as occurred in the control arm of this experiment), could be restabilized by angiotensin II blockade such that they no longer lost podocytes autonomously. If this concept is correct, then we might expect that discontinuing angiotensin II blockade after a period of stabilization would reverse the stabilization effect. We therefore used the same protocol to stabilize glomeruli for 8 weeks after DT-induced podocyte depletion, at which time a kidney biopsy was performed to confirm equal injury in both groups. At this point, drug was discontinued in one group. Figure 5 shows that on discontinuing angiotensin II blockade, proteinuria immediately increased in association with an increase in urine podocyte biomarkers including the urine podocin/nephrin mRNA ratio. Increased podocyte loss and worse histological appearance in the drug-discontinued group was confirmed by quantification of the podocyte complement (Figure 5).

Thus, angiotensin II blockade started 5 days after initiation of podocyte injury did not affect the initial acute phase podocyte loss following DT injection. However, angiotensin II blockade markedly reduced the autonomous chronic phase of podocyte loss in association with protection of kidneys from progression to ESKD. Furthermore, if angiotensin II blockade treatment was stopped, glomeruli became destabilized again with recurrence of progressive podocyte loss leading to glomerulosclerosis.

**Figure 1 | Autonomous progression following initial podocyte depletion in the hDTR Fischer 344 rat model.** (a) Representative histology for sequential kidney biopsies. From left to right, micrographs are from control and at 4, 8, and 13 weeks after diphtheria toxin (DT) injection. Upper panels show podocytes identified using glomerular epithelial protein 1 (GLEPP1)/peroxidase. Lower panels show Masson's trichrome staining. Arrows show sclerosed and partially sclerosed glomeruli. The boxes show higher magnification of an individual glomerulus. (b) Quantification of histology using WT1-positive podocyte nuclear number. (c) Percentage of glomerular tuft area that was GLEPP1 positive. (d) Time course of urine protein/creatinine ratio, urine podocyte (podocin and nephrin) mRNA excretion, and urine podocin/nephrin mRNA ratio. Note that the urine protein/creatinine ratio was increased after injury and continued at a high level until end-stage kidney disease at 13 weeks (top). In the acute phase (first 21 days after injury), both podocin and nephrin mRNA levels were increased. During the chronic phase of progression (from 4 to 13 weeks), urine podocin mRNA remained elevated throughout the time course of progression showing autonomous podocyte loss over the entire time course. In contrast, nephrin mRNA levels were not correspondingly increased during the chronic phase, and thus the urine podocin/nephrin mRNA ratio is a marker of the chronic progression phase.  $n = 8$ ,  $*P < 0.05$  and  $**P < 0.01$ , as assessed by Kruskal-Wallis test and then Scheffe's test. The serum creatinine values in these animals were  $2.52 \pm 0.36$  mg/dl vs.  $0.43 \pm 0.06$  mg/dl in the normal controls ( $P < 0.01$ , as assessed by Student's *t*-test). NS, nonsignificant.







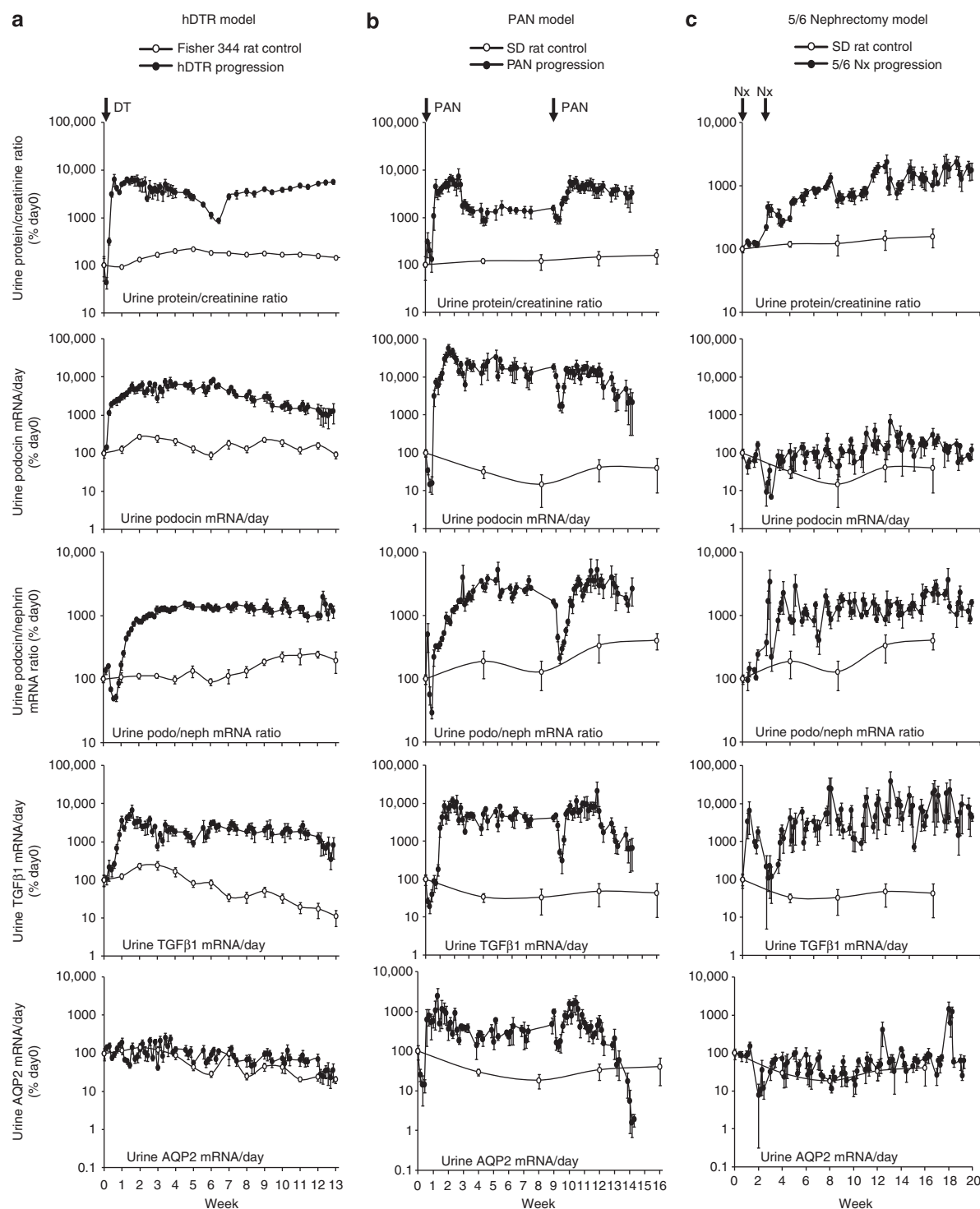
**Figure 2 | The effect of variable amounts of initial podocyte depletion in hDTR rat model. (a)** Glomerular podocyte number measured by WT1-positive nuclei immunofluorescence staining in severe ( $n = 5$ ), moderate ( $n = 13$ ), mild ( $n = 5$ ), and control ( $n = 4$ ) groups at 4 and 8 weeks.  $^{\#}P < 0.05$ . **(b)** Percentage of glomerular tuft area that was glomerular epithelial protein 1 (GLEPP1) positive in severe, moderate, mild, and control groups at 4 and 8 weeks. **(c)** Mean daily urine protein/creatinine ratio in severe, moderate, mild, and control groups. **(d)** Mean daily urine podocin mRNA excretion in severe, moderate, mild, and control groups. **(e)** Mean daily urine nephrin mRNA excretion in severe, moderate, mild, and control groups. **(f)** Mean daily urine podocin/nephrin mRNA ratio in severe, moderate, mild, and control groups.  $^*P < 0.05$  and  $^{**}P < 0.01$  vs. each groups,  $^{##}P < 0.01$  vs. each time point, as assessed by Kruskal-Wallis test and then Scheffe's test. NS, nonsignificant.

### Blood pressure reduction and podocyte depletion

To assess the effect of blood pressure reduction on podocyte loss, we first used the classical triple therapy regimen (hydralazine at 40 mg/kg/day, reserpine at 1.3 mg/kg/day, and hydrochlorothiazide at 17 mg/kg/day) delivered in the drinking water. Drug treatment was again started 5 days after initiation of injury. Figure 6 shows that triple therapy significantly reduced blood pressure in the acute phase but became ineffective in the chronic phase when blood pressure in treated and non-treated groups both increased in parallel.

Proteinuria, histological parameters, renal function, and urine podocyte mRNA levels were not protected in the drug-treated group.

In a second experiment (Figure 7), we used the renin inhibitor aliskiren, started 5 days after initiation of injury, which was delivered by osmotic minipump for 8 weeks. The effect of aliskiren (25 mg/kg/day) was compared with losartan (50 mg/kg/day) and to a combination of losartan and aliskiren. Aliskiren alone reduced systolic blood pressure to the normal range throughout the study period. However,

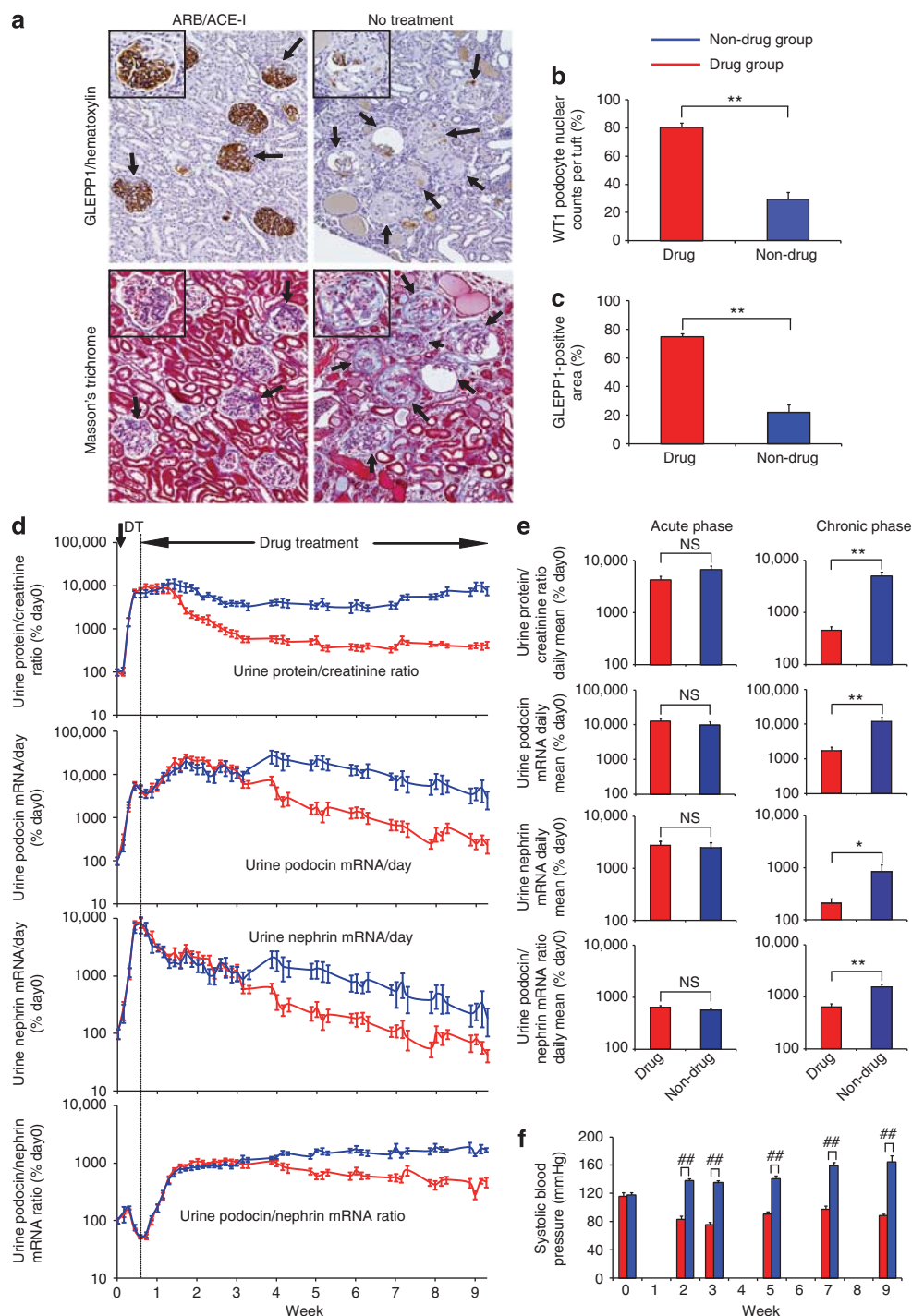


**Figure 3 | Urine biomarkers data comparing the hDTR model (a), the PAN model (b) and the 5/6 nephrectomy model (c).** Comparison of urine biomarkers for three models of progression, including the hDTR Fischer 344 rat model (a), the PAN Sprague–Dawley (SD) rat model (b), and the 5/6 nephrectomy SD rat model (c). Biomarkers include urine protein/creatinine ratio, urine podocin, and podocin/nephrin (podo/neph) ratio as measures of podocyte loss and stress, transforming growth factor (TGF)β1 mRNA as a marker of the profibrotic process, and aquaporin 2 (AQP2) as a kidney tubular cell marker. During progression, similar events were observed in all three model systems, compatible with the conclusion that similar processes were taking place during progression. Experimental data are shown in closed circles and control data are shown in open circles for different rat strains used. DT, diphtheria toxin; hDTR, human diphtheria toxin receptor; PAN, puromycin aminonucleoside; Nx, nephrectomy.

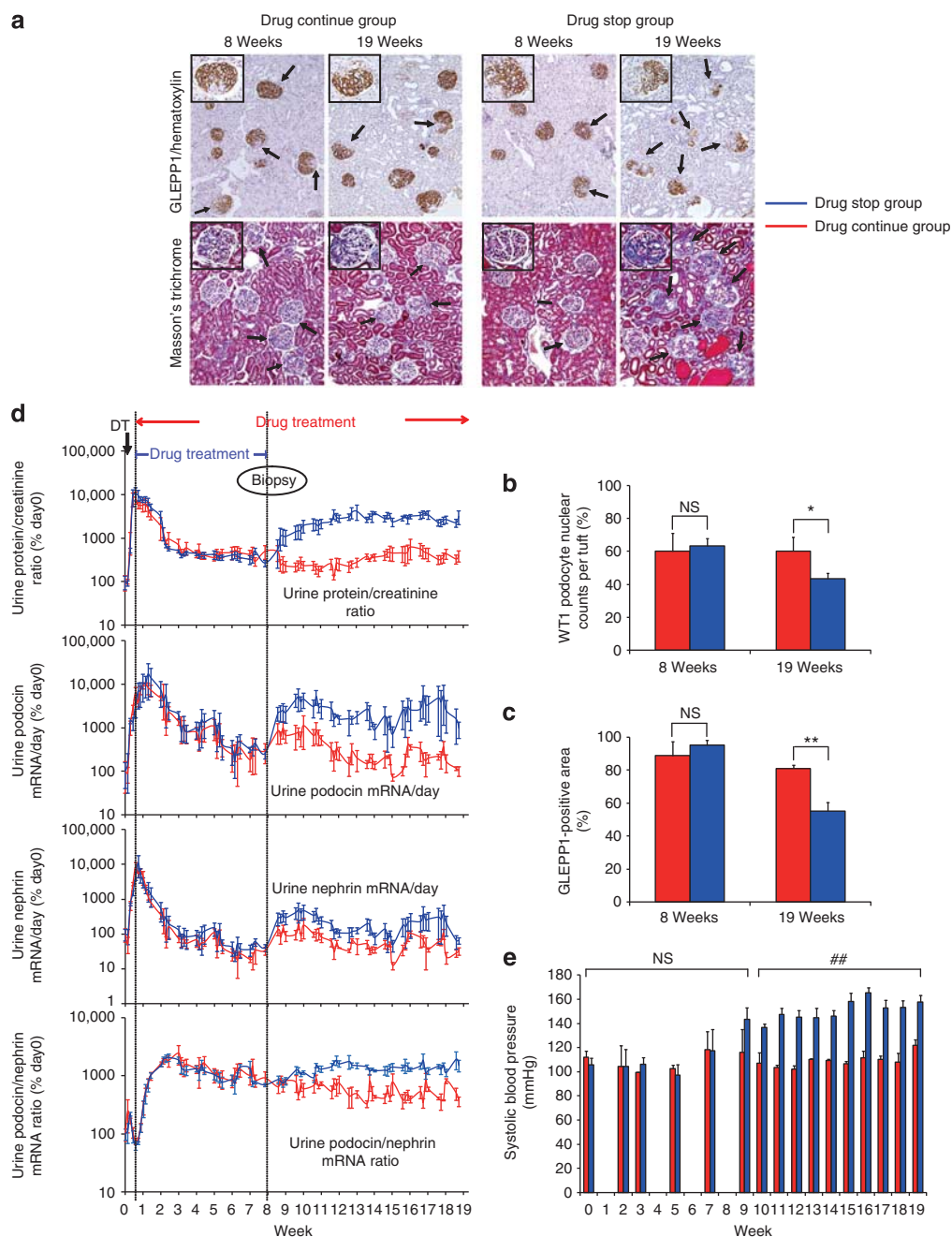
aliskiren did not reduce proteinuria or urine podocyte markers, and did not significantly reduce glomerular podocyte loss or glomerulosclerosis. In contrast, losartan

alone reduced blood pressure by a similar amount to aliskiren alone, but also reduced proteinuria, the rate of podocyte loss as assessed by urine mRNA markers, the degree of podocyte



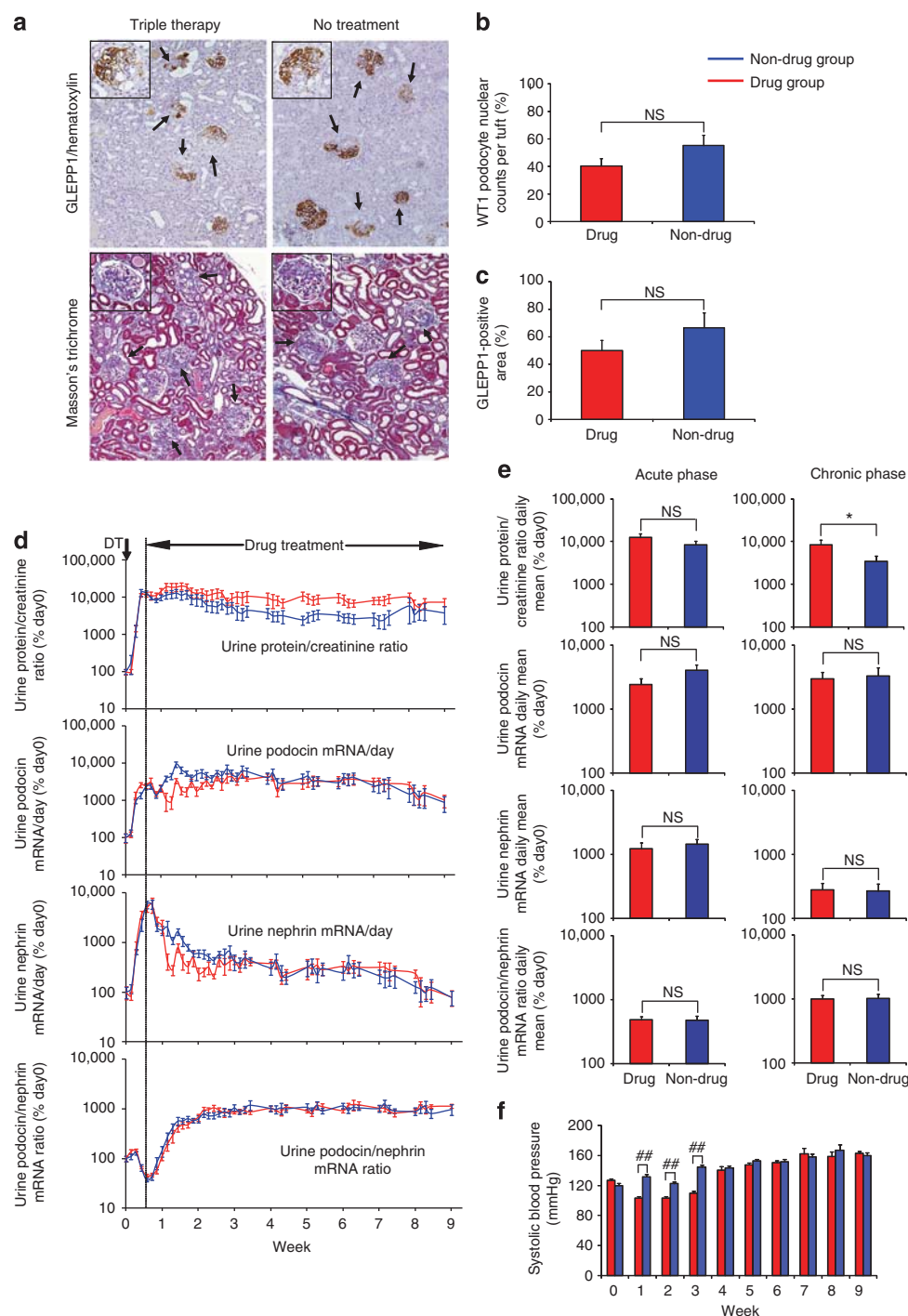


**Figure 4 | Prevention of autonomous podocyte depletion by angiotensin II blockade.** (a) Representative histology with or without angiotensin II blockade in the hDTR rat model. Upper panels show podocytes identified using glomerular epithelial protein 1 (GLEPP1)/peroxidase. Lower panels show Masson's trichrome staining. Arrows show sclerosed and partially sclerosed glomeruli. The boxes show higher magnification of an individual glomerulus. (b) Quantification of histology with or without angiotensin II blockade by WT1-positive nuclei immunofluorescence staining. (c) Percentage of glomerular tuft area that was GLEPP1 positive. (d) Time course of urine protein/creatinine ratio, urine podocyte (podocin and nephrin) mRNA excretion, and urine podocin/nephrin mRNA ratio with or without angiotensin II blockade. Note that the drug-treated group showed reduced proteinuria by about 5 days after the start of drug treatment. In the acute phase (the first 21 days after injury), urine podocyte mRNA (podocin and nephrin) excretion was not significantly different; however, in the chronic phase (after 21 days), urine podocyte mRNAs and urine podocin/nephrin mRNA ratio were significantly reduced in the drug-treated group. (e) Acute and chronic phase analysis of the urine protein/creatinine ratio, urine podocyte mRNAs, and urine podocin/nephrin mRNA ratios. (f) Time course of systolic blood pressure with or without angiotensin II blockade.  $n = 7$  per group,  $*P < 0.05$  and  $**P < 0.01$ , as assessed by Student's  $t$ -test.  $^{##}Z > 3$  as assessed by Z-statistical analysis. The serum creatinine values in non-drug-treated animals were  $2.60 \pm 0.14$  mg/dl vs.  $0.73 \pm 0.04$  mg/dl in the drug-treated animals ( $P < 0.01$ , as assessed by Student's  $t$ -test). ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker; NS, nonsignificant.



**Figure 5 | Discontinuation of angiotensin II blockade causes glomerular destabilization in the hDTR rat model.** (a) Representative histology for angiotensin II blockade continued for 19 weeks or discontinued after 8 weeks. Upper panels show podocytes identified using glomerular epithelial protein 1 (GLEPP1)/peroxidase. Lower panels show Masson's trichrome staining. Arrows show sclerosed and partially sclerosed glomeruli. The boxes show higher magnification of an individual glomerulus. (b) Quantification of histology measured by WT1-positive podocyte number. (c) Percentage of glomerular tuft area that was GLEPP1 positive. (d) Time course of urine protein/creatinine ratio, urine podocin and nephrin mRNA excretion, and urine podocin/nephrin mRNA ratio. Note that proteinuria was reduced throughout the time course in the continuous drug group, but increased after drug discontinuation. Urine podocyte mRNAs (podocin and nephrin) and urine podocin/nephrin mRNA ratio also increased after drug discontinuation. (e) Time course of systolic blood pressure.  $n = 3$  for the drug continuation group,  $n = 4$  for the drug discontinuation group. For d and e, a two-sample Z-statistic was used to compare the mean levels in the two arms of the study. The number and percentage of  $P$ -values below 0.05 before and after drug discontinuation, respectively, were as follows: systolic blood pressure, 0/6 (0%) and 9/9 (100%); urine protein/creatinine ratio, 4/35 (11.4%) and 40/40 (100%); urine podocin mRNA, 1/35 (2.9%) and 38/40 (95%); urine nephrin mRNA, 2/35 (5.7%) and 23/40 (58%); and urine podocin/nephrin mRNA ratio, 2/35 (5.7%) and 33/40 (82.5%). If there was no difference between the two groups, on average 5% of  $P$ -values will be below 0.05. For 35 independent measurements, the 95% range of the percentage of  $P$ -values below 0.05 is 0–14%. Thus, all results above are consistent with there being no difference between the two groups of rats before discontinuation of drug, and a strong difference after discontinuation of drug. \* $P < 0.05$  and \*\* $P < 0.01$ , as assessed by Student's  $t$ -test. ## $Z > 3$  as assessed by Z-statistical analysis. DT, diphtheria toxin; NS, nonsignificant.





**Figure 6 | Blood pressure reduction in the acute phase using triple therapy did not prevent autonomous podocyte depletion in the hDTR rat model.** (a) Representative histology with or without triple therapy (hydralazine, reserpine, and hydrochlorothiazide). Upper panels show podocytes identified using glomerular epithelial protein 1 (GLEPP1)/peroxidase staining. Lower panels show Masson's trichrome staining. Arrows show sclerosed and partially sclerosed glomeruli. The boxes show higher magnification of an individual glomerulus. (b) Quantification of histology with or without triple therapy showing no difference in glomerular podocyte number by WT1-positive nuclear immunofluorescence. (c) Percentage of glomerular tuft area that was GLEPP1 positive. (d) Time course of urine protein/creatinine ratio, urine podocyte (podocin and nephrin) mRNA excretion, and urine podocin/nephrin mRNA ratio. Proteinuria, urine podocyte mRNAs (podocin and nephrin), and urine podocin/nephrin mRNA ratio showed no difference between drug-treated and control groups. (e) Acute and chronic phase analysis of the urine protein/creatinine ratio, urine podocyte (podocin and nephrin) mRNA excretion, and urine podocin/nephrin mRNA ratio. (f) Time course of systolic blood pressure with or without triple therapy showing reduction of blood pressure only in the acute phase.  $n = 7$  for the drug-treated group and  $n = 8$  for the non-drug-treated group,  $*P < 0.05$ , as assessed by Student's  $t$ -test.  $^{##}Z > 3$  as assessed by Z-statistical analysis. The serum creatinine values in non-drug-treated animals were  $1.19 \pm 0.17$  mg/dl vs.  $1.47 \pm 0.28$  mg/dl in the drug-treated animals,  $P = 0.40$ , as assessed by Student's  $t$ -test. DT, diphtheria toxin; NS, nonsignificant.

depletion as assessed by podocyte counting in histological sections, and the degree of glomerulosclerosis.

The failure of aliskiren to reduce proteinuria and prevent podocyte loss and glomerulosclerosis in these experiments could reflect the fact that aliskiren has a >100-fold lower affinity for rat renin than human renin.<sup>34</sup> Therefore, renin inhibition within rat glomeruli in these experiments may simply not have been adequate to prevent critical concentrations of intraglomerular angiotensin II from being generated, while at the same time being adequate to lower systemic blood pressure. Aliskiren may also have affected brain renin activity, which has recently been shown to have a role in regulating systemic blood pressure.<sup>35</sup> However, these experiments do demonstrate conclusively that reduction of systemic blood pressure alone into the normal range was not sufficient to prevent autonomous podocyte loss once glomeruli had been destabilized by a critical degree of podocyte injury.

#### **Is preventing podocyte loss a major mechanism by which angiotensin II prevents progression in the hDTR model?**

Figure 8a and b demonstrate that following threshold depletion of ~30% of podocytes, there was a strong linear relationship between further podocyte depletion and the development of both glomerular and interstitial fibrosis ( $R^2 = 0.77$  and  $0.61$ , respectively). Figure 8c and d demonstrate that the relationship between podocyte loss and both glomerulosclerosis and interstitial fibrosis was not significantly altered irrespective of whether animals had been treated with angiotensin II blockade. These data are therefore compatible with the conclusion that the protective effect of angiotensin II blockade in this model system occurred predominantly through preventing podocyte loss rather than through a podocyte depletion-independent mechanism.

#### **Does the high podocin/nephrin mRNA ratio in urine reflect an altered ratio within the renal cortex during progression, and is this affected by angiotensin II blockade?**

Glomerular nephrin is well known to be downregulated in association with chronic glomerular diseases such as diabetic glomerulosclerosis.<sup>36–42</sup> Therefore, we evaluated whether in the hDTR model system nephrin is relatively downregulated during progression in the renal cortex. The podocin/nephrin mRNA ratio in renal cortex of progressing animals ( $n = 7$ ) was significantly higher than the podocin/nephrin mRNA ratio in control rats ( $n = 5$ ;  $5.15 \pm 0.44$  vs.  $1.14 \pm 0.11$ ,  $P < 0.001$ ). Angiotensin II blockade ( $n = 8$ ) decreased the cortical podocin/nephrin mRNA ratio ( $2.60 \pm 0.45$  vs.  $5.15 \pm 0.44$ ,  $P < 0.001$ ), but did not return it to baseline ( $2.60 \pm 0.45$  vs.  $1.14 \pm 0.11$ ,  $P = 0.03$ ). Furthermore, the nephrin/GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA ratio in renal cortex was decreased 18-fold in progressing rats compared with control ( $0.001 \pm 0.0003$  vs.  $0.018 \pm 0.002$ ,  $P < 0.01$ ), whereas the podocin/GAPDH mRNA ratio was decreased only 3-fold ( $0.007 \pm 0.001$  vs.  $0.021 \pm 0.002$ ,  $P < 0.01$ ) in association with progression.

These data are compatible with the conclusion that nephrin mRNA expression is preferentially reduced compared with podocin mRNA expression in renal cortex during progression, resulting in a high podocin/nephrin mRNA ratio in both renal cortex and in urine of progressor rats, and that angiotensin II has a role in driving these events.

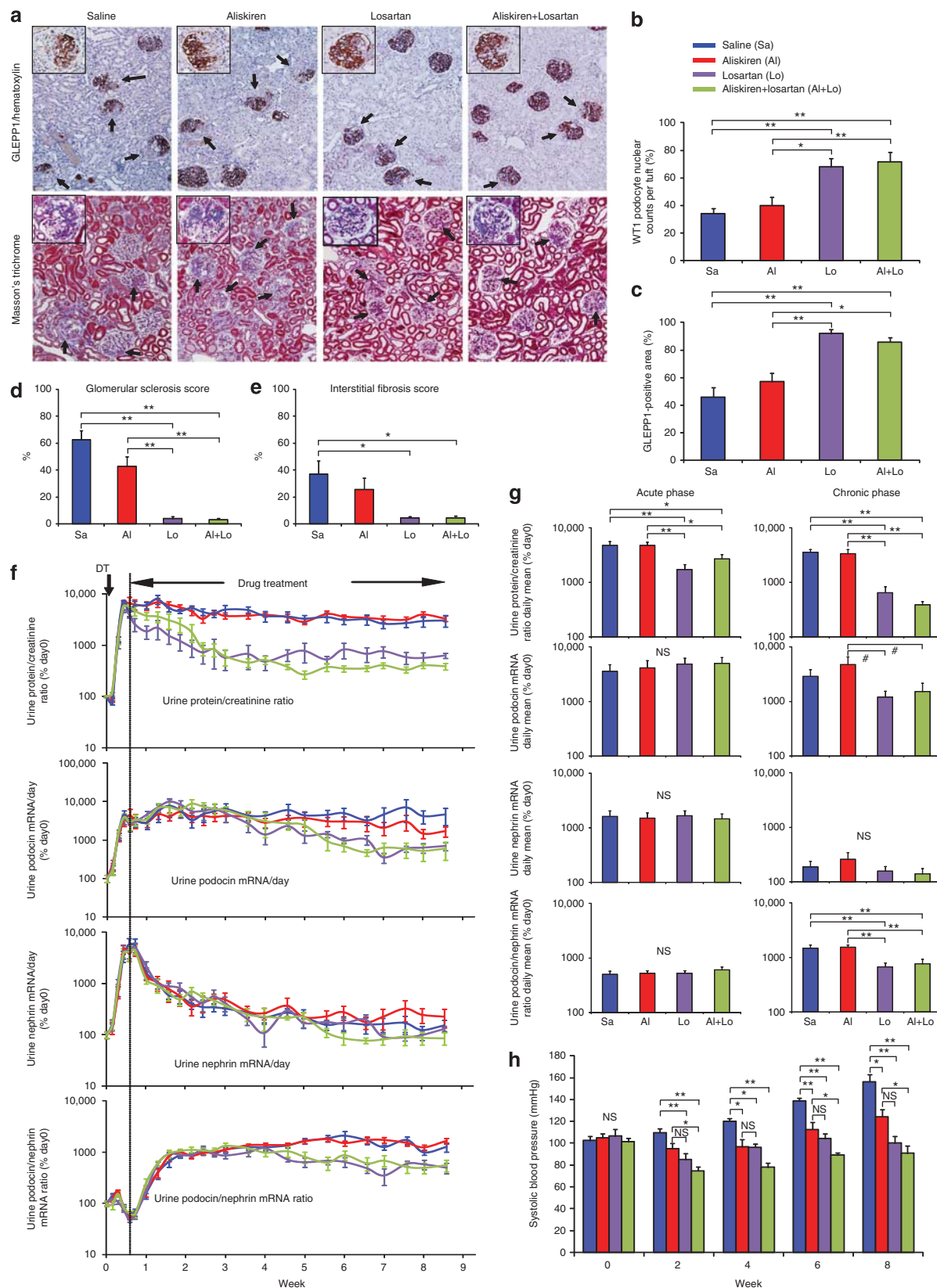
#### **DISCUSSION**

We demonstrate that following a critical degree of podocyte depletion, the glomerulus can become destabilized, resulting in continued persistent podocyte loss without further insult until glomeruli become globally depleted of podocytes (autonomous progression to ESKD). This autonomous podocyte loss process was prevented by angiotensin II blockade. Once destabilized by a critical degree of podocyte depletion, continuous angiotensin II blockade was required for glomeruli to remain stable. Stopping angiotensin II blockade resulted in renewed podocyte loss and further progressive loss of kidney function. The protective effect of angiotensin II blockade was not due to blood pressure reduction alone, as reducing blood pressure to the normal range, even by the renin inhibitor aliskiren, did not prevent persistent podocyte loss and progression. Thus, angiotensin II blockade, probably functioning directly on the podocyte, appears to be necessary for optimal prevention of podocyte loss following initial podocyte injury. These concepts, illustrated diagrammatically in Figure 9, outline how a critical degree of glomerular injury from any cause can be amplified by angiotensin II-dependent further podocyte depletion, and how this process can be monitored noninvasively through urine mRNA biomarkers. These concepts also provide a mechanistic explanation for the ‘hyperfiltration hypothesis’ of Brenner *et al.*<sup>43</sup>

The two major functions of the glomerulus are filtration of blood and volume control via the juxtaglomerular apparatus. Changes in intraglomerular dynamics caused by podocyte depletion and protein leak into the filtrate result in activation of the renin–angiotensin system, as is well described in the nephrotic syndrome. Mechanistically, angiotensin II would cause increased intraglomerular pressure, as well as direct effects on glomerular cells through angiotensin receptors. For example, the podocyte has both AT1 and AT2 receptors;<sup>44–46</sup> overexpression of AT1 receptors by podocytes causes glomerulosclerosis;<sup>45</sup> in podocyte cell culture systems, angiotensin II modifies cytoskeletal structure and the podocyte phenotype in an epithelial–mesenchyme transformation-like manner, and causes downregulation of nephrin expression;<sup>46–49</sup> and nephrin expression is well documented to be downregulated in chronic glomerular diseases.<sup>36–42</sup> We also demonstrate that progression was associated with relative cortical nephrin mRNA downregulation in comparison with podocin mRNA, and this was ameliorated by angiotensin II blockade. These data all support the concept that one mechanism driving autonomous podocyte loss is an effect of angiotensin II in causing a podocyte phenotypic switch that results partly in nephrin

downregulation. The time delay between angiotensin II-induced hemodynamic effects (reduced proteinuria/blood pressure) and reduced podocyte loss in urine, and the fact

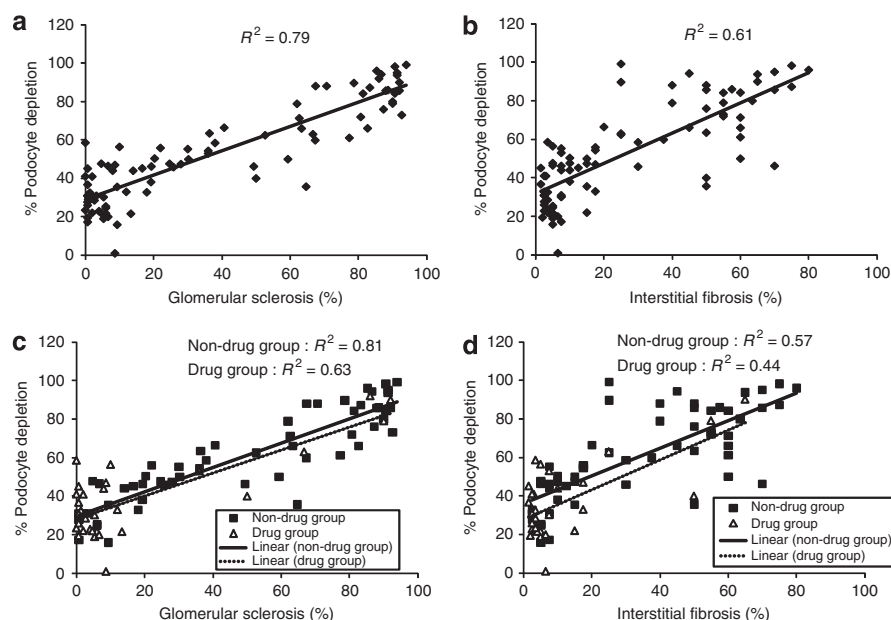
that lowering systemic blood pressure to the normal range did not reduce the rate of podocyte loss, also support the hypothesis that reducing blood pressure and even reduced



intraglomerular pressure (as judged by reduced proteinuria) may not itself be sufficient to prevent podocyte loss from the destabilized glomerulus. Additional effects of angiotensin II blockade in modulating the podocyte phenotype may also be required. Stopping angiotensin II blockade resulted in rapid rebound destabilization of glomeruli and triggered further podocyte loss, compatible with the clinical concept that maintaining angiotensin II blockade long term in patients with previously destabilized

glomeruli can prevent or retard subsequent glomerular and interstitial fibrosis.

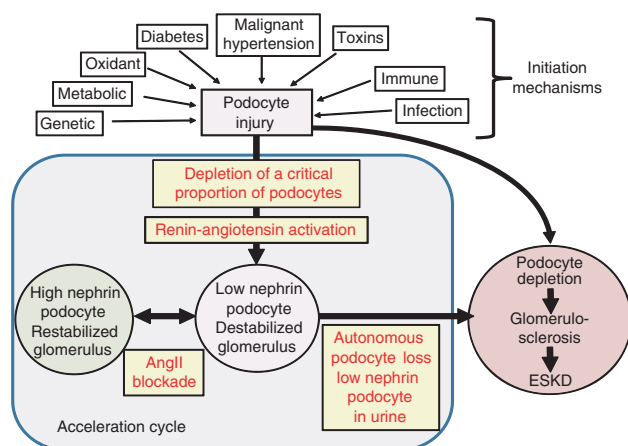
The angiotensin II-dependent mechanism described in this report would likely be different from the 'podocyte damage damages podocyte' concept reported by Ichikawa *et al.*,<sup>13</sup> who used a chimeric mouse model to demonstrate that damage to a podocyte results in damage to its neighboring podocyte ('vicious cycle of podocyte-to-podocyte damage'). Matsusaka *et al.*<sup>14</sup> used podocyte-specific



**Figure 8 | Relationship between glomerular sclerosis or interstitial fibrosis score and percentage depletion of the WT1 podocyte nuclear count.** (a) Relationship between glomerular sclerosis score and percentage depletion of WT1 podocyte nuclear count for all animals ( $n = 87$ ,  $R^2 = 0.79$ ,  $P < 0.001$ ). (b) Relationship between interstitial fibrosis score and percentage depletion of WT1 podocyte nuclear count for all animals ( $n = 87$ ,  $R^2 = 0.61$ ,  $P < 0.001$ ). (c) Relationship between glomerular sclerosis score and percentage depletion of podocyte count with (closed squares and thick line) or without (open triangles and thin line) angiotensin II blockade. Angiotensin II blockade caused reduced podocyte loss but did not change either the slope or the coordinates of the correlation (non-drug group:  $n = 57$ ,  $R^2 = 0.81$ ,  $P < 0.001$ ; drug group:  $n = 30$ ,  $R^2 = 0.63$ ,  $P < 0.001$ ). (d) Relationship between interstitial fibrosis score and percentage depletion of WT1 podocyte nuclear count with (closed squares and thick line) or without (open triangles and thin line) angiotensin II blockade. Angiotensin II blockade reduced podocyte loss but did not change the slope of the relationship (non-drug group:  $n = 57$ ,  $R^2 = 0.57$ ,  $P < 0.001$ ; drug group:  $n = 30$ ,  $R^2 = 0.44$ ,  $P < 0.001$ ).

**Figure 7 | Blood pressure reduction throughout the time course using aliskiren (delivered at 25 mg/kg/day by osmotic minipump) did not reduce proteinuria or prevent autonomous podocyte depletion.** In contrast, similar blood pressure reduction using the angiotensin II receptor blocker losartan reduced proteinuria and podocyte depletion. (a) Representative histology in saline, aliskiren, losartan, and aliskiren + losartan groups. Upper panels show podocytes identified using glomerular epithelial protein 1 (GLEPP1)/peroxidase staining. Lower panels show Masson's trichrome staining. Arrows show sclerosed and partially sclerosed glomeruli. The boxes show higher magnification of an individual glomerulus. Color coding is shown by the box at upper right for the saline, aliskiren, losartan, and aliskiren + losartan groups. (b) Quantification of glomerular podocyte number by WT1-positive nuclear immunofluorescence. (c) Percentage of glomerular tuft area that was GLEPP1 positive. (d) Quantification of glomerular sclerosis score. (e) Quantification of interstitial fibrosis score. (f) Time course of urine protein/creatinine ratio, urine podocyte (podocin and nephrin) mRNA excretion, and urine podocin/nephrin mRNA ratio. Aliskiren alone did not reduce proteinuria or urine podocyte markers. In contrast, losartan alone and aliskiren + losartan reduced proteinuria and urine mRNA markers. (g) Acute and chronic phase analysis of the urine protein/creatinine ratio, urine podocyte mRNAs, and urine podocin/nephrin mRNA ratio. (h) Time course of systolic blood pressure showing significant reduction of blood pressure to the normal range throughout the time course in aliskiren, losartan, and aliskiren + losartan group rats.  $n = 8$  (saline and aliskiren groups),  $n = 6$  (losartan and aliskiren + losartan groups). \* $P < 0.05$  and \*\* $P < 0.01$ , as assessed by Kruskal-Wallis test and then Scheffe's test. # $Z > 2$  as assessed by Z-statistical analysis. The serum creatinine values in these four groups were  $2.64 \pm 0.29$ ,  $2.14 \pm 0.13$ ,  $1.29 \pm 0.07$ , and  $1.33 \pm 0.10$  mg/dl ( $P < 0.01$ , saline vs. losartan and saline vs. aliskiren + losartan,  $P < 0.05$ , aliskiren vs. losartan, as assessed by Kruskal-Wallis test and then Scheffe's test). DT, diphtheria toxin; NS, nonsignificant.





**Figure 9 | Diagrammatic summary of the glomerular destabilization and progression hypothesis.** Many factors can cause podocyte injury. If as a result of this initiating injury there is a critical level of podocyte depletion, then the glomerulus can become destabilized such that further podocyte loss occurs independent of the initiating event and driven by the renin-angiotensin system until glomeruli become globally depleted of podocytes (end-stage kidney disease, ESKD). This progression-accelerating mechanism can be retarded or prevented by angiotensin II (AngII) blockade (glomerular restabilization), but discontinuation of angiotensin II blockade results in relapse to the destabilized glomerular phenotype with recurrence of podocyte loss. These events can be monitored noninvasively by measuring the podocyte mRNA products in urine.

deletion of the AT1 receptor in a mouse toxin model to suggest that direct effect of angiotensin II on podocytes may not be responsible for podocyte injury. However, these studies do not exclude an effect through podocyte AT2 receptors, and the experimental design for these studies used pretreatment of mice by losartan 5 days before toxin injection, raising the possibility that angiotensin II blockade could have modified glomerular filtration of the 60 kDa toxin and thereby reduced access of the toxin to podocytes.<sup>14</sup> Thus, the precise mechanism by which angiotensin II drives podocyte injury and loss remains to be unequivocally established.

Angiotensin II is capable of driving the profibrotic process through different mechanisms.<sup>50</sup> Our data are compatible with the conclusion that the predominant protective effect of angiotensin II blockade occurred through preventing podocyte depletion and its downstream consequences. Mechanistically, this could occur through downstream effects of podocyte loss-induced proteinuria in driving interstitial fibrosis as described by Eddy,<sup>51</sup> protein leak into the periglomerular and interstitial space as a driver of interstitial fibrosis as described by Kriz and LeHir,<sup>10</sup> and through TGF $\beta$  expression in relation to these processes.<sup>52</sup>

In the hDTR and PAN model systems, all glomeruli become simultaneously depleted of podocytes. The clinical syndrome revealed by these models resembles rapidly progressive glomerular diseases such as those that are seen

clinically in association with crescentic nephritis and other conditions wherein podocytes become depleted from glomeruli over relatively short periods of time. For example, in crescentic nephritis, podocytes are lost into the crescent and change their phenotype;<sup>53–56</sup> in HIV-associated nephropathy, podocytes switch their phenotype to lose podocyte markers and presumably podocyte function;<sup>57,58</sup> and in severe inflammatory conditions, podocytes are lost in increased quantities into the urine.<sup>13,14</sup> However, in many human glomerular diseases, the underlying progression process occurs over months or years, and the glomerular lesions are typically focal and segmental (affecting different glomeruli to different degrees at different times), such as those that were observed in the 5/6 nephrectomy model in this report and in the aging model in *ad libitum*-fed rats.<sup>59</sup> We expect that the same mechanism and time course of autonomous podocyte loss from destabilized glomeruli will occur for an individual glomerulus, or a capillary loop within a glomerulus, in chronic glomerular diseases, as was observed for the whole population of glomeruli in the hDTR model. Thus, the difference in clinical phenotype of slow vs. rapid progression is probably due to the same underlying biology taking place in single glomeruli or parts of glomeruli, as opposed to a large proportion of glomeruli, at any point in time.

The potential clinical utility of urine podocyte mRNAs (and particularly the urine podocin/nephrin mRNA ratio) to noninvasively monitor events taking place in the glomerulus in real time is supported by the data shown. It is clear that these studies, which use a limited pastiche of urine mRNAs to describe the podocyte, can potentially provide a rich source of information about glomerular biology, which may include both the rate of podocyte loss and podocyte stress, as well as podocyte replacement from parietal epithelial stem cells and other sources. If these markers can be reliably harnessed, they could provide a new paradigm for noninvasive monitoring and management of glomerular diseases.

## MATERIALS AND METHODS

### Animals

All animal studies were approved by the University of Michigan Committee on Use and Care of Animals. All drug treatment was started 5 days after intravenous DT injection. We performed daily urine collection for 3 weeks, followed by 4 days/week urine collection until the end of each study. Systolic blood pressure,<sup>60</sup> serum and urine creatinine, and urine protein were measured as described previously.<sup>11,33</sup>

### hDTR Fischer 344 rat model

For standard protocol, heterozygous 100 g hDTR transgenic rats received 25 ng/kg DT injected intravenously in normal saline (0.9%) containing 0.1 mg/ml rat albumin as a carrier. Kidney biopsies were performed to remove approximately one-tenth of one kidney at 4 and 8 weeks, and kidneys were harvested at 13 weeks. Timed urine samples were also collected from control Fischer 344 rats ( $n = 5$ ) 2 days per week for 13 weeks. Data are shown in Figure 3a as the mean weekly value. In a second experiment to obtain a range of

podocyte depletion, the DT doses used were 25–40 ng/kg intravenously ( $n=12$ ) or intraperitoneally ( $n=11$ ) as previously described.<sup>31</sup> These rats ( $n=27$ ) were divided into three groups based on the degree of podocyte depletion at 4 weeks (control, mild <20%, moderate 20–60%, and severe >60%).

### Angiotensin II blockade model

Rats receiving standard DT protocol were assigned to two groups: drug group, receiving enalapril, 10 mg/kg/day (cat. no. E6888, Sigma, St Louis, MO), and losartan, 50 mg/kg/day (provided by Merck, Rahway, NJ) in drinking water, and non-drug group, not receiving the drug. Animals in the non-drug-treated group reached ESKD by 9 weeks after DT injection. In the second experiment, standard protocol rats were assigned to the two groups: drug continuation group and drug discontinuation group. Both groups received enalapril and losartan in drinking water for 8 weeks. Following a kidney biopsy, the drug continuation group continued using the drug, whereas the drug discontinuation group stopped using the drug. Rats were killed 19 weeks after DT injection.

### Triple therapy treatment model

Rats receiving standard protocol were assigned to the two groups: drug group, receiving hydralazine (40 mg/kg/day; cat. no. H1753, Sigma), reserpine (1.3 mg/kg/day; cat. no. R0875, Sigma), and hydrochlorothiazide (17 mg/kg/day; cat. no. H4759, Sigma) in drinking water, and non-drug group, receiving water alone. Rats were killed at 9 weeks after DT injection. In initial studies, adding labetalol up to 80 mg/kg/day to the triple therapy regimen did not lower blood pressure further (data not shown).

### Aliskiren treatment model

Rats receiving standard protocol were assigned to the four groups: saline, aliskiren alone, losartan alone, and aliskiren + losartan. Drug-treated groups received aliskiren 25 mg/kg/day (provided by Novartis International AG, Basel, Switzerland) or saline both delivered by osmotic minipump (Alzet 2ml4 minipump (Alzet Osmotic Pumps, Cupertino, CA) implanted subcutaneously under anesthetic and replaced after 4 weeks), or losartan 50 mg/kg/day in drinking water, or aliskiren by minipump and losartan in the drinking water. Rats were euthanized at 9 weeks after DT injection.

### Protein overload model

Heterozygous 100 g hDTR Tg Fischer 344 rats received a daily injection of 1 g/day fatty acid-free bovine serum albumin (cat. no. A8806, Sigma) intraperitoneally for 4 days with daily urine collection preceding and during 4 days of albumin injections. Rats were euthanized at 4 days after albumin injection.

### Puromycin aminonucleoside SD rat model

Male SD rats (100 g) received 20 mg/100 g PAN (cat. no. P7130, Sigma) intraperitoneally. After 8 weeks, a kidney biopsy was done, and then a second PAN injection (12.5 mg/100 g) was given intravenously. Rats were killed 69–105 days after the first PAN injection when they reached ESKD. Timed urine samples were collected from control SD rats ( $n=5$ ). Urine was collected 2 days per month for 16 weeks, with the monthly mean value used as shown in Figure 3b.

### 5/6 Nephrectomy rat model

Two-thirds (extended poles) of the right kidney of SD male rats were removed when they reached ~100 g of weight. One week after the

partial right nephrectomy, the whole of left kidney was removed. Urine and blood pressure measurements were recorded as described for the hDTR model above. Rats were euthanized at 18 weeks after 5/6 nephrectomy. Timed urine samples were collected from control SD rats ( $n=5$ ). Urine was collected 2 days per month for 16 weeks, with the monthly mean value used as shown in Figure 3c.

### RNA from urine sediments and cortex

Urine was collected overnight (average 15 h) and cortex was dissected into small pieces after being perfused using cold phosphate-buffered saline using methods previously described, except that total urine pellet and cortex RNA was isolated using the protocol of the RNeasy Mini Kit (cat. no. 74106; Qiagen, Hilden, Germany).<sup>31</sup>

### Quantitative real-time PCR

Quantification of the absolute nephrin, podocin, TGF $\beta$ 1, aquaporin 2, Slc7a13, and GAPDH mRNA abundance was performed using the 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using TaqMan Fast Universal PCR Master Mix, with sample complementary DNA in a final volume of 10  $\mu$ l per reaction. TaqMan Probes (Applied Biosystems) used were as follows: Rat homolog for NPHS1 (nephrin) spanned exons 20–21 (cat. no. Rn00575235\_m1); rat homolog for NPHS2 (podocin) spanned exons 3–4 (cat. no. Rn00709834\_m1); rat TGF $\beta$ 1 spanned exons 1–2 (cat. no. Rn00572010\_m1); rat aquaporin 2 spanned exons 1–2 (cat. no. Rn00563755\_m1); rat Slc7a13 spanned exons 3–4 (cat. no. Rn01764598\_m1); and rat GAPDH was within exon 3 (cat. no. Rn99999916\_s1). All data were from 2  $\mu$ g sample complementary DNA. Standard curves were constructed using these serially diluted standards.<sup>33</sup>  $C_T$  values were used to analyze mRNA levels from standard curve using the SDS 2.2.2 software (Applied Biosystems).

### Histological analysis

Paraformaldehyde/lysine/periodate-perfused and fixed kidney, followed by paraffin embedding for sectioning, was used as described previously.<sup>11,33</sup> WT1-positive podocyte nuclei per glomerular tuft were counted as previously described using the following modification.<sup>61</sup> WT1 primary antibody and Cy3-labeled secondary antibody were used to identify podocyte nuclei in 4,6-diamidino-2-phenylindole-stained sections so that podocyte nuclei appeared as a shocking pink color that facilitated identification in biopsy samples, where blood components remain in glomerular capillaries and cause nonspecific immunofluorescent signals. GLEPP1-positive tuft area was estimated as previously described method.<sup>11,33,61</sup> Glomerular and interstitial fibrosis scoring was measured using 3- $\mu$ m Masson's trichrome-stained sections. Mean glomerular fibrosis score was estimated from 50 consecutive glomerular cross-sections. Interstitial fibrosis score was estimated using the Metamorph imaging program to measure the percentage of the non-glomerular cortex area that stained blue in  $\times 10$  images.

### Statistical methods

All results were presented as means  $\pm$  s.e.m., except where otherwise noted. Differences between two groups were tested using Student's  $t$ -test, and among more than two groups by Kruskal–Wallis test. When the Kruskal–Wallis test was significant, Scheffe's test was carried out for *post hoc* analysis. A two-sample  $Z$ -statistic was used to compare the mean levels in blood pressure data. Correlations between parameters were compared by single regression analysis.  $P<0.05$  and  $Z>2$  was considered to be statistically significant.

## DISCLOSURE

All the authors declared no competing interests.

## ACKNOWLEDGMENTS

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## SUPPLEMENTARY MATERIAL

**Figure S1.** Proteinuria *per se* does not cause elevated urine podocyte mRNA levels.

**Figure S2.** Progression was induced in Sprague-Dawley rat by injection of puromycin aminonucleoside (PAN) at time 0 and after kidney biopsy at 8 weeks.

**Figure S3.** Progression in the 5/6 nephrectomy rat model. Supplementary material is linked to the online version of the paper at <http://www.nature.com/ki>

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